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(54) Title: AMPHIPHILIC DERIVATIVES OF GUANIDINE

(57) Abstract

Guanidine-based amphiphiles are provided that are non-toxic to the host mammal, especially a human host. The amphiphiles are used to produce liposomes useful as carriers for delivering macromolecules intracellularly.

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AMPHIPHILIC DERIVATIVES OF GUANIDINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/157,727, filed November 24, 1993, which is a continuation-in-part of USSN 07/991,935, filed December 17, 1992, which disclosures are herein incorporated by reference.

INTRODUCTION

FIELD OF THE INVENTION

This invention relates to nitrogen containing amphiphiles for use in the preparation of liposomes and other lipid-containing carriers of pharmaceutical substances, including nucleic acids used in gene therapy.

BACKGROUND OF THE INVENTION

Liposomes are one of a number of lipid-based materials used as biological carriers and have been used effectively as carriers in a number of pharmaceutical and other biological situations, particularly to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcriptional factors and other cellular vectors into a variety of cultured cell lines and animals. Successful clinical trials have examined the effectiveness of liposome-mediated drug delivery for targeting liposome-entrapped drugs to specific tissues and specific cell types. See, for example, U.S. patent No. 5,264,618, which describes a number of techniques for using lipid carriers, including the preparation of liposomes and pharmaceutical compositions and the use of such compositions in clinical situations. However,

while the basic methodology for using liposome-mediated vectors is well developed, improvements in the materials used in the methods, both in terms of biocompatability and in terms of effectiveness of the carrier process, are still desirable.

In particular, the expression of exogenous genes in humans and/or various commercially important animals will ultimately permit the prevention and/or cure of many important diseases and the development of animals with commercially important characteristics. Genes are high molecular weight, polyanionic molecules for which carrier-mediated delivery usually is required for DNA transfection of cells either *in vitro* or *in vivo*. Therefore it is of interest to develop lipid transfection vectors which will enhance both the delivery and the ultimate expression of the cloned gene in a tissue or cell of interest. Since in some instances a treatment regimen will involve repeated administration of a gene (or other pharmaceutical product), it also is of interest that the lipid carriers be nontoxic to the host, even after repeated administration.

RELEVANT LITERATURE

Literature describing the use of liposomes as carriers for DNA include the following: (Friedmann (1989), supra; Brigham, et al., (1989) Am. J. Med. Sci., 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA expression vectors (Nabel et al. (1990), supra; Wolff et al.

(1990) Science, 247:1465-1468). Direct injection of transgenic material into tissue produced only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra). Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract) have reported *in vivo* transfection restricted to lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. See also Stribling et al. Proc. Natl. Acad. Sci. (USA) 89:11277-11281 (1992) which reports the use of liposomes as carriers for aerosol delivery of transgenes to the lungs of mice and Yoshimura et al. Nucleic Acids Research (1992) 20:3233-3240.

Cationic lipid carriers have been shown to mediate intracellular delivery of plasmid DNA (Felgner, et al., Proc. Natl. Acad. Sci. USA (1987) 84:7413-7416); mRNA (Malone, et al., Proc. Natl. Acad. Sci. USA (1989) 86:6077-6081); and purified transcription factors (Debs, et al., J. Biol. Chem. (1990) 265:10189-10192), in functional form.

SUMMARY OF THE INVENTION

Non-toxic, novel, amphiphilic derivatives of guanidine are provided as are the methods of their use. The amphiphiles are capable of forming complexes with nucleic acids, and other biological compounds, and the nucleic acid complexes are capable of transforming mammalian cells. The amphiphiles of the invention are non-toxic even when subjected to endogenous enzymatic processes.

DESCRIPTION OF SPECIFIC EMBODIMENTS

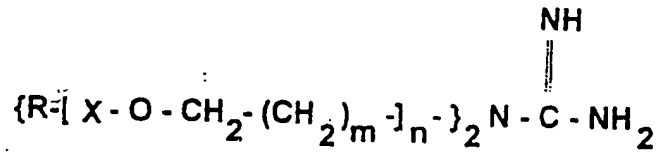
Metabolizable amphiphilic derivatives of guanidine are provided which are useful as carriers for biologically active molecules, such as antibiotics or nucleic acids used in cell transformation processes. The use of the amphiphilic materials as nucleic acid carriers is described in detail, since the compositions prepared using the amphiphiles are particularly efficacious for this purpose. However, the amphiphiles are also useful in standard drug delivery regimens, such as for the delivery of antibiotics to the lungs of a patient. In particular, complexes of the amphiphiles with DNA (for the transformation of cells in mammalian tissues) give rise to reduced amounts of toxic cleavage products when subject to the metabolic degradation process.

The invention in particular is directed to amphiphilic derivatives of guanidine which are nontoxic themselves and which yield by-products, such as those produced by enzymatic cleavage, which are nontoxic to a host organism or which are identical to substances endogenous to a host organism. These amphiphiles thus offer the advantage that they can readily be used in humans, since they can be used repeatedly without the accumulation of toxic by-products.

It will be apparent that the cations of the invention must be present in association with one or more anions, e.g., hydroxide, chloride, or bromide ions or more complex organic anions or bases. The particular anion associated with an amphiphilic cation is not critical to the formation or utility of the amphiphilic cation and may exchange (in whole or part) for other anions during use of the composition. Accordingly, the amphiphilic compounds of the invention are described in this specification generally in terms of the cation without reference to

any particular anion. However, a number of specific examples are given, as well as general guidance for selection of anions. For human administration, chloride is the preferred anion; also acceptable are bromide or other physiologically acceptable anions including acetate, succinate and citrate. The cations are either nontoxic themselves, and/or they yield by-products, for example, enzymatic cleavage products, which are nontoxic to a host organism or which are endogenous to a host organism. Generally, both the original lipids and their degradation products are nontoxic to a host organism.

The invention particularly relates to novel nitrogen-containing amphiphilic compounds having the formula:

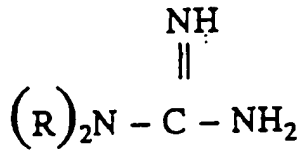


wherein each R independently is a straight-chain, aliphatic hydrocarbyl group of 5 to 29 carbon atoms inclusive, each X is $-CH_2-$ or -CO-, each m is an integer from 0 to 7 inclusive and each n is zero or 1, with the proviso that when n is 1, the total number of carbon atoms in R and $-(CH_2)_m-$ is at least 10, and when n is zero, each R independently is a straight-chain, aliphatic hydrocarbyl group of at least 11 carbon atoms inclusive. Preferred derivatives of the above formula I are those wherein n is 1. Also preferred are those compounds of formula I wherein m is from 1 to 5 inclusive, particularly 1. Also preferred are those derivatives wherein each R independently has from 13 to 23 carbon atoms inclusive. The R groups are saturated or are unsaturated having one or more ethylenically unsaturated linkages and are suitably the same or are different from each other. Also

preferred are those derivatives wherein X is -CO-, in which case illustrative R groups together with the -CO- group to which it is attached (i.e., R-CO-) include lauroyl, myristoyl, palmitoyl, stearoyl, linoleoyl, eicosanoyl, tricosanoyl and nonacosanoyl (derived from the fatty acids of the corresponding name: lauric, myristic, etc.). Alternatively, X can be $-CH_2-$. When given system names for the R groups alone, the corresponding names of the hydrocarbyl group derived from lauric acid is undecyl; from myristic acid, tridecyl; from palmitic acid, pentadecyl; from stearic acid, heptadecyl; from linoleic acid, cis,cis-8,11-heptadecydienyl; from eicosanoic acid, nonadecyl; from tricosanoic acid, dicosanyl; and from hemicosanoyl, nonacosanyl. This grouping of R groups is preferred when n is 1. When n is 0, R is preferably the entire hydrocarbyl portion of a fatty alcohol, such as a lauryl, stearyl, or myristyl group.

In the modification of the amphiphilic compounds of formula I wherein n is zero, the compounds are of the formula

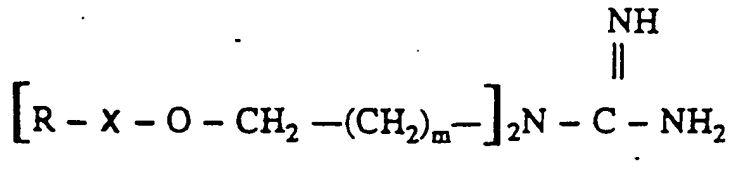
(II)



wherein R has the previously stated meaning. Illustrative of such compounds are N,N-distearylguanidine, alternatively named amidinodioctadecylamine, N,N-dilaurylguanidine, alternatively named amidinodidodecylamine, and N,N-dimyristylguanidine, alternatively named amidinodi(tetradecyl)amine. Other illustrative compounds of the above formula II will be apparent from the formula and the above meaning of R.

In the modification of the amphiphiles of formula I wherein n is 1, the compounds are of the formula

(III)



wherein R, X and m have the previously stated meanings. Such compounds are illustrated by N,N-di[2-(palmitoyloxy)ethyl]guanidine, alternatively named amidino-di[2-(hecadecanoyloxy)ethyl]amine, by N,N-di[2 (oleoyloxy)ethyl]guanidine, alternative named amidino[2-(9-octadecenoyloxy)ethyl]amine, and by N,N-- di[6-(stearoyloxy)hexyl]guanidine, alternatively named amidinodi[6-(octadecanoyloxy)hexyl]amine. Other illustrative compounds of formula III will be apparent from the formula and the above meanings of R and m.

For convenience, the amphiphilic compounds of the invention represented by formula II can be viewed as N,N-di-R-guanidine derivatives and the compounds represented by formula III can be visualized as N,N-di(R-carboxyalkyl)guanidine derivatives, wherein R has the previously stated meaning, although as previously stated the derivatives are not necessarily prepared from guanidine. In general, the compounds of formula III are preferred over the compounds of formula II.

There are a number of synthetic techniques in the art that have been developed for the synthesis of guanidinium compounds. A general synthesis that can be used to produce compounds of the invention involves the conversion of a dialkanolamine to a diacyl derivative (after protecting the amine), deprotection of the amine, and reaction of the resulting secondary amine with cyanamide in base

to provide the desired product. The initial dialkanolamine can be obtained commercially (diethanolamine is readily available in quantity and is inexpensive) or can be synthesized by standard dialkylation reactions for the production of secondary amines from hydroxy-protected omega-hydroxyalkylhalides. Omega-hydroxyalkylhalides are themselves available from the corresponding alpha,omega-dihydroxyalkanes, which can be readily prepared from cycloalkenes by oxidation (e.g., with ozone) and reduction. The acyl groups are available from the acid halides (or anhydrides) of the corresponding carboxylic acids, which, as previously indicated, are preferably fatty acids and thus available commercially.

On the other hand, several of the general methods are not suitable for producing the amphiphiles of the present invention. For example, the reaction of secondary aliphatic amines with bromocyanide will form the corresponding cyanidamine, which then has been reported to form a substituted guanidine on reaction with ammonium chloride; however, the second step of this procedure did not work for making the compounds described herein. It has also been reported that aliphatic amines can also be reacted with cyanamide in either acetic acid or n-butanol to produce the corresponding guanidine derivative, although the reaction in acetic acid did not work for making the compounds described herein. Finally, reaction of primary or secondary amines with 3,5-dimethylpyrazole-1--carboxamidine nitrate in aqueous solution for several days has been shown to form guanidines, although this method did not work for making the compounds described herein. Nevertheless, either the method outlined in general above or detailed examples provided in more detail in the Examples section below will be sufficient to produce any of the guanidinium compounds within the scope of the

invention.

The cationic lipids of the invention are typically used as carriers for various biological molecules, such as antibiotics or nucleic acids. In particular, the cationic lipids can be used alone or combined with other lipids in formulations for the preparation of lipid vesicles or liposomes for use in intracellular delivery systems. Uses contemplated for the lipids of the invention include transfection procedures corresponding to those presently known that use amphiphilic lipids, including those using commercial cationic lipid preparations, such as Lipofectin ™, and various other published techniques using conventional cationic lipid technology and methods. The cationic lipids of the invention can be used in pharmaceutical formulations to deliver therapeutic agents by various routes and to various sites in an animal body to achieve a desired therapeutic effect.

Because such techniques are generally known in the art, background information and basic techniques for the preparation of pharmaceutical compositions containing lipids will not be repeated at this time. A reader unfamiliar with this background information is referred to the publications under the heading Relevant Literature above and further to U.S. Patent No. 5,264,618. This last-cited patent describes a number of therapeutic formulations and methods in detail, including examples of the use of specific cationic lipids (different from those described here) that can be followed in detail by substituting the cationic lipids of the present invention for those described in the patent. Compositions of the present invention will minimally be useable in the manner described in the patent, although operating parameters may need to be modified in order to achieve optimum results, using the specific information provided for compounds of the

invention in this specification along with the knowledge of a person skilled in the arts of lipid preparation and use.

The lipids of the present invention are particularly useful and advantageous in the transfection of animal cells by genetic material. Additionally, since these compositions are non-toxic even when subjected to host enzymatic reactions, the compositions provide a number of advantages in the area of low toxicity when compared to previously known cationic lipids. These and other advantages of the invention are discussed in detail below. The remainder of this discussion is directed principally to selection, production, and use parameters for the cationic lipids of the present invention that may not immediately be apparent to one of ordinary skill in the art.

Particularly where it is desirable to target a lipid-DNA complex to a particular cell or tissue, a lipid mixture used as a carrier can be modified in a variety of ways. By a lipid mixture is intended a formulation prepared from the cationic amphiphile of the invention, with or without additional agents such as steroids, and includes liposomes, interleaved bilayers of lipid, and the like. Steroids, e.g. cholesterol or ergosterol, can be used in combination with the cationic amphiphiles when used to prepare mixtures. In some embodiments, the lipid mixture will have from 0-67 mole percent steroid, preferably about 33 to 50 mole percent steroid. A lipid-DNA complex is the composition obtained following combination of DNA and a lipid mixture. Non-lipid material (such as biological molecules being delivered to an animal or plant cell or target-specific moieties) can be conjugated through a linking group to one or more hydrophobic groups, e.g. using alkyl chains containing from about 12 to 20 carbon atoms, either prior

or subsequent to vesicle formation. Various linking groups can be used for joining the lipid chains to the compound. Functionalities of particular interest include thioethers, disulfides, carboxamides, alkylamines, ethers, and the like, used individually or in combination. The particular manner of linking the compound to a lipid group is not a critical part of this invention, as the literature provides a great variety of such methods. Alternatively, some compounds will have hydrophobic regions or domains which will allow for their association with the lipid mixture without covalent linking to one or more lipid groups.

For the most part, the active compounds to be bound to the lipid mixture ligands or receptors capable of binding to some biological molecule of interest is present in the target cell. A ligand can be any compound of interest which can specifically bind to another compound, referred to as a receptor, the ligand and receptor forming a complementary pair. The active compounds bound to the lipid mixture can vary widely, from small haptens (molecular weights of about 125 to 2,000) to antigens which will generally have molecular weights of at least about 6,000 and generally less than about 1 million, more usually less than about 300,000. Of particular interest are proteinaceous ligands and receptors that have specific complementary binding partners on cell surfaces. Illustrative active compounds include chorionic gonadotropin, encephalon, endorphin, luteinizing hormone, morphine, epinephrine, interferon, ACTH, and polyiodothyronines and fragments of such compounds that retain the ability to bind to the same cell-surface binding partners that bind the original (non-fragment) molecules.

The number of targeting molecules (either ligand or receptor) bound to a lipid mixture will vary with the size of the liposome, the size of the molecule, the

binding affinity of the molecule to the target cell receptor or ligand, and the like. Usually, the bound active molecules will be present in the lipid mixture in from about 0.05 to 2 mole percent, more usually from about 0.01 to 1 mole percent based on the percent of bound molecules to the total number of molecules available in the mixture for binding.

The surface membrane proteins which bind to specific effector molecules (usually soluble molecules in the external environment of the cell) are referred to as receptors. In the present context, receptors include antibodies and immunoglobulins since these molecules are found on the surface of certain cells. However, since antibodies are generally used to bind liposomes to receptor molecules on target cells, the antibodies and immunoglobulins bound to a liposome containing a cationic lipid of the invention can also be considered to be ligands. The immunoglobulins may be monoclonal or polyclonal, preferably monoclonal. Usually the immunoglobulins will be IgG and IgM, although the other immunoglobulins may also find use, such as IgA, IgD, and IgE. The intact immunoglobulins may be used or only fragments thereof, such as Fab, $F(ab')_2$, F_d, or F_v fragments as well as a complete light or heavy chain.

For antibodies used as cell-targeting ligands, antibodies of interest are those that bind to surface membrane antigens such as those antigens comprising the major histocompatibility complex, particularly the HLA-A, -B, -C and -D. Other surface antigens include thy-1,leu-5, and Ia.

The cationic amphiphiles are particularly useful as carriers for anionic compounds, particularly polyanionic macromolecules such as nucleic acids. Where the amphiphiles are intended for use in vivo, particularly in vivo in

humans, or where it is necessary to use the amphiphiles repeatedly, it is important to screen the carriers for those which are metabolized to non-toxic by-products and which themselves are not toxic or those which are eliminated from the body without degradation. The elimination of such cationic amphiphiles from tissues can be demonstrated in animal experiments. An animal, such as a mouse, can be administered one or more doses of material containing between 0.5 and 10 pmole of the lipid to be tested, complexed with an active component (such as DNA) if desired. At various times after administration, the animals are sacrificed, tissues taken, total lipids extracted using an appropriate solvent extraction system, and the total lipid analyzed for the particular cationic lipid or its partial degradation product using, for example, HPLC.

The cationic amphiphiles are positively charged, and a tight charge complex can be formed between a cationic lipid carrier and a polyanionic nucleic acid, resulting in a lipid carrier-nucleic acid complex which can be used directly for systemic delivery to a mammal or mammalian cell. Where delivery is via aerosolization, the charge complex will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:lipid carrier complex has been deposited in the lung following intranasal or intraoral delivery of the aerosolized complex.

To evaluate the efficacy of a particular cationic amphiphile for use as a nucleic acid carrier in an aerosolization process, as well as to determine the optimum concentrations of lipid carrier-nucleic acid complexes, involves a two-step process. The first step is to identify lipid carriers and the concentration of lipid carrier-nucleic acid complexes that do not aggregate when the components

are combined or during the significant agitation of the mixture that occurs during the nebulization step. The second step is to identify among those lipids that do not aggregate those complexes that provide for a high level of transfection and transcription of a gene of interest in target cells in the lung. These techniques are described in WO/US PCT/US92/11008 filed December 17, 1992, which disclosure is hereby incorporated by reference.

As an example, a reporter gene CAT (which encodes chloramphenicol acetyltransferase) can be inserted in an expression cassette and used to evaluate each lipid carrier composition of interest. The DNA:lipid carrier complexes are mixed in solutions which do not themselves induce aggregation of the DNA:lipid carrier complexes, such as sterile water. The expression cassette (DNA) is mixed together with each of the lipid carriers to be tested in multiple different ratios, ranging as an example from 4:1 to 1:10 (micrograms of DNA to nanomoles of cationic lipid or total lipid, if a lipid mixture is present). Examination of the stability of the resulting mixtures provides information concerning which ratios result in aggregation of the DNA:lipid carrier complexes and are therefore not useful for use in vivo, and which complexes remain in a form suitable for aerosolization. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confer the highest level of transgene expression in vivo. For example, for aerosol-based transfection, the optimal DNA:lipid carrier ratios for lipid mixtures such as N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-triethylammonium chloride(DOTMA):dioleoylphosphatidylethanol-amine(DOPE) (the components of this mixture being present in a 1:1 weight ratio) and dimethyl dioctadecyl ammonium bromide (DDAB):Cholesterol (1:1) are 1 to

1. For O-ethyl egg phosphatidylcholine (E-EPC) or especially O-ethyl dimyristoylphosphatidylcholine (E-DMPC) in a 1:1 weight ratio with cholesterol, the DNA:lipid carrier ratio is preferably in the range of from 1.5:1 to 2:1.

If the cationic amphiphile is used for injection, then it need be evaluated only for whether it is effective for transfection of a target cell.

Particular cells can be targeted by the use of particular cationic lipids for preparation of the lipid-mixture carriers, for example, by the use of E-DMPC to target lung cells preferentially, or by modifying the amphiphiles to direct them to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be employed, to target a cell associated with a particular surface protein. A particular ligand or antibody can be conjugated to the cationic amphiphile in accordance with conventional techniques, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer or by providing a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art.

The various lipid carrier-nucleic acid complexes wherein the lipid carrier is a liposome are prepared using methods well known in the art. Mixing conditions can be optimized by visual examination of the resultant lipid-DNA mixture to establish that no precipitation occurs. To make the lipid-DNA complexes more visible, the complexes can be stained with a dye which does not itself cause aggregation, but which will stain either the DNA or the lipid. For example, Sudan black (which stains lipid) can be used as an aid to examine the lipid-DNA mixture to determine if aggregation has occurred. Particle size also can be studied

with methods known in the art, including electron microscopy, laser light scattering, Coulter™ counting/sizing, and the like. Standard-size beads can be included as markers for determining the size of any liposomes or aggregates that form. By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to the surface of a lipid carrier preparation, as discussed below. The lipid carrier preparation can also include other substances, such as enzymes necessary for integration, transcription and translation or cofactors. Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types. Generally, the nucleic acid material is added to a suspension of preformed liposomes which may be multi-lamellar vesicles (MLVs) or small unilamellar vesicles (SUVs), usually SUVs formed by sonication. The liposomes themselves are prepared from a dried lipid film that is resuspended in an appropriate mixing solution such as sterile water or an isotonic buffer solution such as 10mM Tris/NaCl or 5 % dextrose in sterile water and sonicated to form the liposomes. Then the preformed lipid carriers are mixed directly with the DNA.

Mixing and preparing of the lipid-DNA complex can be critically affected by the sequence in which the lipid and DNA are combined. Generally, it is preferable (to minimize aggregation) to add the lipid to the DNA at ratios of DNA:lipid of up to 1:2 inclusive (microgram DNA:nanomoles cationic lipid). Where the ratio of DNA:lipid is 1:4 or higher, better results are generally obtained by adding the DNA to the lipid. In either case, mixing should be rapidly achieved by shaking or vortexing for small volumes and by use of rapid mixing systems for large volumes. The lipid carrier and DNA form a very stable

complex due to binding of the negatively charged DNA to the cationic lipid carriers. SUVs find use with small nucleic acid fragments as well as with large regions of DNA (≥250kb).

In preparing the lipid carrier-nucleic acid complex for nebulization, care should be taken to exclude any compounds from the mixing solution which promote the formation of aggregates of the lipid carrier-nucleic acid complexes. Large particles generally will not be aerosolized by the nebulizer, and even if aerosolized would be too large to penetrate beyond the large airways. Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/8 ml solution, and avoiding the use of chelating agents such as EDTA and/or significant amounts of salt, either of which tends to promote macro-aggregation. The preferred excipient is water, dextrose/water or another solution having low or zero ionic strength. Further, the volume should be adjusted to the minimum necessary for deposition in the lungs of the host mammal, while at the same time taking care not to make the solution too concentrated so that aggregates form. Increasing the volume of the solution is to be avoided if possible due to the need to increase the inhalation time for the host animal to accommodate the increased volume. In some cases, it may be preferable to lyophilize the lipid carrier-nucleic acid complexes for inhalation. Such materials are prepared as complexes as described above, except that a cryoprotectant such as mannitol or trehalose is included in the buffer solution which is used for preparation of the lipid carrier-DNA complexes. Any glucose generally included in such a buffer is preferably omitted. The lipid carrier

complex is rapidly freeze-dried following mixing of the lipid and DNA. The mixture can be reconstituted with sterile water to yield a composition which is ready for administration to a host animal.

Where the amphiphiles form liposomes, the liposomes may be sized in accordance with conventional techniques, depending upon the desired size. In some instances, a large liposome injected into the bloodstream of an animal has higher affinity for lung cells as compared to liver cells. Therefore, the particular size range may be evaluated in accordance with any intended target tissue by administering lipid-nucleic acid complexes of varying particle sizes to a host animal and determining the size of particle which provides the desired results.

The cationic amphiphiles complexed with nucleic acid of this invention can be administered in a variety of ways to a host, such as intravenously, intramuscularly, subcutaneously, transdermally, topically, intraperitoneally, intravascularly, by aerosol, following nebulization, and the like. Normally, the amphiphiles will be injected in solution where the concentration of compound bound to or entrapped in the liposome will dictate the amount to be administered. This amount will vary with the effectiveness of the compound being administered, the required concentration for the desired effect, the number of administrations, and the like. In some instances, particularly for aerosol administration, the lipid-DNA complexes can be administered in the form of a lyophilized powder.

Upon administration of the amphiphiles, when a targeting moiety is used, the amphiphiles preferentially bind to a cell surface factor complementary to the compounds bound to the liposome. If no targeting moiety is bound to the liposome, then it binds to cell surface by lipophilic interactions. The liposomes

normally are transferred into the cell by endocytosis.

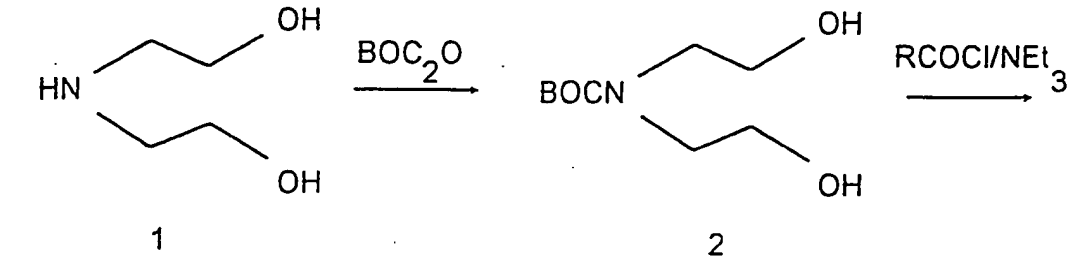
The cationic amphiphiles find use for complexing with nucleic acid or protein for transporting these macromolecules in vivo. The nucleic acid can include DNA, RNA, antisense RNA or other antisense molecules. Cationic amphiphiles that form liposomes also find use in drug delivery, where the drug can be entrapped within the liposome or bound to the outside.

The following examples are offered by way of illustration and not by way of limitation.

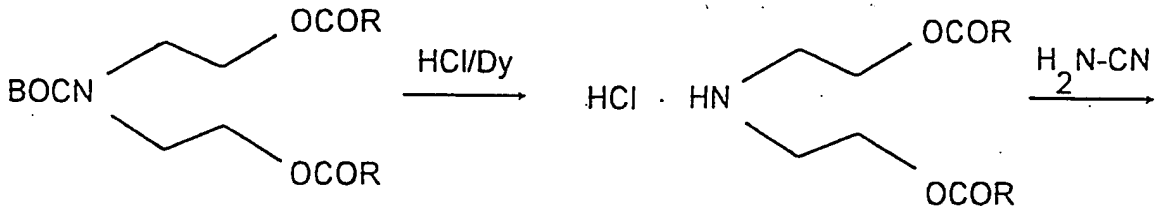
EXAMPLES

Example 1

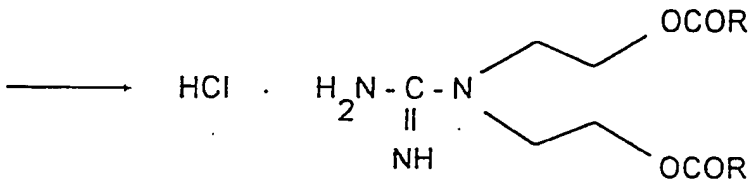
(a) Preparation of N-amidino-O,O-diacyldiethanolamine



1 2



3 4



5

Commercially available diethanolamine was protected on nitrogen using di-tert-butyl-pyrocarbonate, acylated with appropriate acyl chloride, N-BOC protection group was cleaved with 4M HCl in dioxane, resulting hydrochloride salt was reacted with cyanamide in n-BuOH to afford the desired N-amidino-O,O-diacyldiethanolamine.

Example: Synthesis of N-amidino-O,O-dipalmitoyldiethanolamine 5.

N-BOC diethanolamine 2.

To a solution of 10 ml (0.1 mol) diethanolamine in 150 ml of acetonitrile were added 22.8 g (0.105 mol) of di-tert-butyl-pyrocarbonate and mixture was

stirred at R.T. overnight. The resulting solution was evaporated, the residue dissolved in ethyl acetate/hexane (7/3) and passed through a plug of silica. After evaporation of solvent to get 16.4 g (80%) of N-BOC diethanolamine.

N-BOC ester 3.

To a solution of 2.0 g (0.0097 mol) of 2 in 100 ml of CH_2Cl_2 at O°C were added 3.4 ml (0.024 mol) of triethylamine, then in 10 min with stirring were added 6.2 ml (0.02 mol) of palmitoyl chloride. The mixture was stirred at O°C for 30 min, then at R.T. for 45 min. The resulting solution was washed with 10% citric acid (50 mlx2), with 10% aqueous solution of sodium bicarbonate (50 mlx2), dried over $MgSO_4$, filtered, filtrate evaporated on rotavapor and the rest was chromatographed on silica gel using 0-15% EtOAc/Hexane to get 6.4 g (95%) of N-BOC ester 3.

Amino ester 4.

To 3.5 g (0.086 mol) of N-BOC ester 3 were added 20 ml of 4M solution of HCl in dioxane and mixture was stirred at R.T. for 2 hrs. The resulting suspension was evaporated on rotavapor, diluted with ether (50 ml), filtered, washed with ether (25 mlx2) and dried in vacuum to get 3.1 g (97%) of amino ester 4.

Amidino ester 5.

To 0.22 g (0.00036 mol) of amino ester 4 was added 0.5 ml of n-BuOH and 0.022 g (0.00054 mol) of cyanamide. The mixture was stirred at 110°C for 1

hr, diluted with chloroform (10ml) and evaporated on rotavapor. White precipitate formed was washed with ether (10ml x 2), then with water (10ml x 2) on filter and dried in vacuum to get 0.190 g (79%) of amidino ester 5.

(b) Transfection using cationic liposomes containing N-amidino-O,O-dipalmitoyldiethanolamine (ADPDE), N-amidino-O,O-dioleoyldiethanolamine (ADODE), and N-amidinodistearoylamine (ADS).

Liposomes containing ADODE, ADPDE or ADS in a 1:1 molar ratio with cholesterol were tested as DNA carriers for gene transfer and expression in mice. The plasmid used was pZN51. The methods and plasmids used are described in more detail in WO93/24640. The liposomes were in a 10mM stock in 5% dextrose. The liposome:plasmid DNA ratios were screened for the presence of aggregation. Ratios from 1:2 to 1:7 (μg plasmid DNA to nanomoles cationic lipid) were screened. DNA:liposome ratios that did not produce aggregation were then tested in mice. 100 μg of pZN51 was complexed to 500 nanomoles of DDAB:cholesterol liposomes as a positive control and an uninjected mouse served as the negative control (N).

ICR female mice (25 g) were used for the *in vivo* studies. A dose of 100 μg plasmid DNA in 200 μl 5% dextrose in water was injected by tail vein per mouse.

The lung, heart, liver, kidney and spleen were removed after 24 hours. Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH7.8, 5 mM EDTA, and the resulting extract was centrifuged and then subjected to 3 cycles of freeze-thaw and then treated to 65 °C for 20 min. The protein concentration of

lung, heart, liver and kidney extracts were quantitated using a ninhydrin-based protein assay (Bio-Rad, Berkeley, CA), and same amount of total protein from each tissue extract was added in the CAT assay, together with 10 μl of 20 mM acetyl CoA+12 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham)), at 37 °C for 13 hrs.

ADODE:CHOL liposomes in a 1:6 ratio produced the highest levels of CAT activity in the lung, liver and heart. However, CAT activity was lower than that produced by DDAB:CHOL in a 1:5 ratio. There was almost no CAT activity in the kidney and spleen in ADODE:CHOL-treated mice.

ADPDE:CHOL liposomes in a 1:6 ratio produced the highest levels of CAT activity in the lung, heart, liver, kidney and spleen. The level of CAT activity was similar to DDAB:CHOL in a 1:5 ratio in these organs.

ADS:CHOL liposomes in a 1:5 ratio produced the highest levels of CAT activity in the lung and liver. The CAT activity was lower than that produced by DDAB:CHOL at the 1:5 ratio. In heart, spleen and kidney ADS:CHOL produces little to no CAT activity.

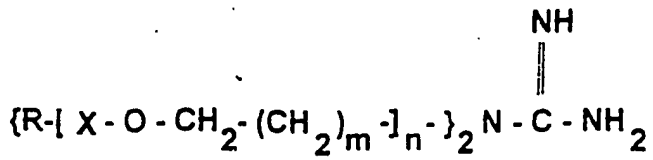
All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A nitrogen-containing amphiphile of the formula:

(I)



wherein each R independently is straight-chain, aliphatic hydrocarbyl group of 5 to 29 carbon atoms inclusive, each X is $-CH_2-$ or -CO-, each m is an integer from 0 to 7 inclusive and each n is zero or 1, with the proviso that when n is 1, the total number of carbon atoms in R and $-(CH_2)_m-$ is at least 10, and when n is zero, each R independently is a straight-chain, aliphatic hydrocarbyl group of at least 11 carbon atoms inclusive.

2. The amphiphile of claim 1 wherein n is zero.

3. The amphiphile of claim 2 wherein each R independently has from 14 to 24 carbon atoms inclusive.

4. The amphiphile of claim 3 wherein R is octadecyl.

5. The amphiphile of claim 3 wherein R is tetradecyl.

6. The amphiphile of claim 1 wherein n is 1.

7. The amphiphile of claim 6 wherein m is 1.

8. The amphiphile of claim 7 wherein X is -CO-.

9. The amphiphile of claim 7 wherein each R independently has from 13 to 23 carbon atoms inclusive.

10. The amphiphile of claim 9 wherein R is pentadecyl.

11. The amphiphile of claim 9 wherein R is 8-heptadecenyl.

12. The amphiphile of claim 6 wherein m is 5.

13. The amphiphile of claim 12 wherein R is heptadecyl.

14. A method of transforming cells in one or more tissues of a mammal, comprising:

contacting said cells with a plurality of complexes comprising an expression cassette and a nitrogen-containing amphiphile of Claim 1, wherein said complexes provide for transmission of cells in at least one tissue of said mammal and are susceptible to endogenous enzymatic cleavage to non-toxic products.

15. A method for transfecting a mammalian cell comprising contacting said cell with a complex comprising a transcription cassette or an expression cassette and a nitrogen-containing amphiphile of Claim 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13428

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 25/26, 25/28; A61K 31/70; C12N 15/00
US CL :424/417; 514/44; 435/172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/417; 514/44; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,264,618 (FELGNER ET AL.) 23 November 1993, see entire document.	1-13 and 15
X	Science, Volume 249, issued 14 September 1990, E.G. Nabel et al , "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall", pages 1285-1288, see page 1286, figure 2.	14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 MARCH 1995	Date of mailing of the international search report 14 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Nathan Fergason Deborah Crouch, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US94/13428

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims1-14, drawn to a nitrogen containing amphiphile and a method of transforming the cells in one or more tissues of a mammal, classified in Class 424, subclass 417 and Class 514, subclass 44.

Group II, claim 15, drawn to a method of transfecting a mammal cell, classified in Class 435, subclass 172.3.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The in vivo transfection of group I and the in vitro transfection of group II require separate and distinct protocols. In addition, neither protocols is required for the implementation of the other protocol.